

Elena Martín-Orozco^a
 Blanca Cárdena^a
 Victoria del Pozo^a
 Belén de Andrés^a
 Mayte Villalba^b
 Soledad Gallardo^a
 María Isabel Rodríguez-García^c
 Mari Carmen Fernández^c
 Juan D. Alché^c
 Rosalía Rodríguez^b
 Pilar Palomino^a
 Carlos Lahoz^a

^a Department of Immunology,
 Fundación Jiménez Díaz,

^b Department of Biochemistry and
 Molecular Biology, Facultad de Ciencias,
 Universidad Complutense, Madrid, and
^c Estación Experimental del Zaidín, Consejo
 Superior de Investigaciones Científicas,
 Granada, Spain

Key Words

Ole e 1
Olea europaea
 Epitope mapping
 Oleaceae pollens, cross-reactivity
 Tree pollens
 Ultrastructural localization
 Olive pollen grain
 ELISA
 Immunoblotting
 Immunocytochemical techniques
 Monoclonal antibody

Ole e 1: Epitope Mapping, Cross-Reactivity with Other Oleaceae Pollens and Ultrastructural Localization

Abstract

Ole e 1 is the major allergen derived from olive tree pollen (*Olea europaea*) and it is composed of two polypeptides with molecular weights (MWs) of 18 and 20 kD. A panel of six monoclonal antibodies (mAbs) has been prepared and used to map antigenic determinants on this molecule. Four epitope determinants have been identified on *Ole e 1*. Using the purified mAbs produced against *Ole e 1*, we have analyzed the common epitope determinants in olive (*O. europaea*) and different Oleaceae pollens: ash (*Fraxinus excelsior*); privet (*Ligustrum vulgare*); lilac (*Syringa vulgaris*), and forsythia (*Forsythia suspensa*). ELISA showed three reactivity groups depending on the recognition of monoclonal antibodies: (1) olive and ash; (2) olive, ash, privet and lilac; and (3) olive, ash, privet, lilac and forsythia. Immunoblotting studies on Oleaceae pollen extracts with these mAbs showed a very similar cross-reactivity pattern. The 18- and 20-kD MW proteins were present in each pollen, except in the case of forsythia. In this case the reactivity pattern was associated with 50- to 55-kD protein bands. This band was recognized by a pool of sera from olive-allergic patients. Finally, ultrastructural localization of *Ole e 1* antigen was performed on the mature olive pollen grain. *Ole e 1* was located in association with dilated endoplasmic reticulum cisternae. Pollen grain walls, nuclei and cytoplasmic organelles were totally devoid of the allergen.

Introduction

The olive tree (*Olea europaea*) is one of the most widely distributed species in the Mediterranean area and its pollen is considered to be one of the major causes of respiratory allergy in this area [1]. Several studies have been carried out by different researchers in order to identify the olive pollen

allergens. Thus, Vela et al. [2] found that the most reactive fractions were those of 65-kD molecular weight (MW), and Blanca et al. [3] assigned the category of major allergen to a polypeptide of MW 15 and 17 kD. Lauzurica et al. [4, 5] reported the identification and isolation of two major allergens of this pollen: *Ole e 1* and *Ole e 2*, corresponding to proteins of 17-19 and 8 kD, respectively. Studies carried out

during the last few years confirmed the reported characteristics of *Ole e 1* and they described its heterogeneous nature [6]. Villalba et al. [7] described the amino acid composition and the N-terminal sequence of *Ole e 1*. Lombardero et al. [8] obtained several monoclonal antibodies against *O. europaea* antigens and developed a two-site solid-phase RIA for the *Ole e 1* quantitation. Obispo et al. [9] also detected the cross-reactivity between pollens of the Oleaceae family. Finally, the complete primary structure of *Ole e 1* was determined recently by Villalba et al. [10] and they report that this major allergen contains a single polypeptide chain of 145 amino acid residues with a calculated molecular mass of 16,331 Daltons.

On the other hand, although partial immunochemical identity among molecules in the same or different allergenic extracts has also been demonstrated in different reports [11–13], relatively few studies on the cross-reactivity between olive pollen and other species have been reported. Vela et al. [2] described common antigenic fractions for *Lolium perenne* and *O. europaea* by crossed electrophoresis, although no cross-allergenicity was found. Bousquet et al. [1] and Kernerman et al. [14] reported a high degree of cross-reactivity between olive (*O. europaea*), ash (*Fraxinus americana*), privet (*Ligustrum vulgare*) and a common bush (*Phillyrea angustifolia*). Finally, Baldo et al. [15] demonstrated allergenic cross-reactivity between olive, privet, rye grass and couch grass pollen components. They also reported that allergenic histograms for olive and privet pollens are remarkably similar and major allergenic components of 19, 20 and 40 kD are present in both species. The cross-reactivity between different crude allergen preparations constitutes an important problem in the diagnosis and treatment of allergic diseases. The application of monoclonal antibody (mAb) technology in this field may be useful to study the specificity of allergenic proteins in crude extracts, and the cross-reactivity from different allergenic sources [16].

In our study we analyzed the epitope determinants of *Ole e 1* and the cross-reactivity of olive with different Oleaceae pollens like privet (*L. vulgare*), ash (*Fraxinus excelsior*), lilac (*Syringa vulgaris*) and forsythia (*Forsythia suspensa*) by using mAbs against *Ole e 1*.

Cellular localization of allergens in the pollen grain of some allergenic species has been studied at the structural level [17–21]. The interest in this type of study is based on the connection between biochemical results and morphological characteristics of the cell. They can offer information about where allergens are stored, or where the synthesis of these molecules may take place. In this report, we also state the ultrastructural localization of the *Ole e 1* antigen using immunocytochemical techniques.

Materials and Methods

Patient Sera

Sera were collected from 10 allergic patients with a clinical history of seasonal allergic pollinosis (rhinitis and/or asthma) and positive skin test for olive pollen crude extract. All patient sera were screened by Phadebas RAST (Pharmacia, Uppsala, Sweden) or by ELISA and showed specific IgE binding to olive crude extract.

Extraction of Pollens

Pollens of *O. europaea*, *F. excelsior*, *L. vulgare*, *S. vulgaris* and *F. suspensa* were obtained from Allergon AB (Välinge, Sweden). The crude extracts were prepared according to the procedure described previously [4]. Briefly, 5 g of pollen were defatted with diethyl ether. After drying, 200 ml of 0.01 M ammonium bicarbonate were added and the suspension was extracted for 4 h at 4°C with continuous stirring. After centrifugation at 12,000 g for 20 min at 4°C, the supernatant was filtered through a 0.22- μ m membrane (Sartorius, Göttingen, Germany) and freeze-dried. Protein determinations were carried out using the method described by Bradford [22].

Ole e 1 Purification

The purification procedures have been described previously by Villalba et al. [7]. The olive crude extract was dissolved in 0.25 M ammonium bicarbonate (pH 8.0) and loaded onto a Sephadex G-75-fine column (18 \times 800 mm) equilibrated in the same buffer. The 18- to 20-kD allergenic fractions were pooled and lyophilized. A reverse-phase HPLC step on a μ Bondapak C-18 column (7.9 \times 300 mm) was used to purify this allergen, using a Beckman system with an acetonitrile gradient in 0.1% trifluoroacetic acid. The eluent was continuously monitored at both 214 and 280 nm wavelengths. The purity of the allergen was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) as described by Laemmli [23]; and by amino acid analysis in a Durrum autoanalyzer.

Preparation of Monoclonal Antibodies

Eight-week-old female BALB/c mice (Iffa Credo, Barcelona, Spain) were immunized intraperitoneally with 50 μ l saline containing 100 μ g of *O. europaea* crude extract emulsified in PBS in complete Freund's adjuvant (Difco, Detroit, Mich., USA). Boosting injections with the same amount of antigen and incomplete Freund's adjuvant (Difco) were given on days 15 and 30. On the 37th day, the mouse sera were screened and selected by ELISA for the presence of antibodies against *Ole e 1* allergen using this pure antigen to coat ELISA wells. Mice with a high titer (>1:12,800) received a final intraperitoneal injection. After 4 days the mice were killed, and their spleen cells fused with P3-NS/1-Ag 4-1 (NP3) myeloma cells according to the method described by Köhler and Milstein [24]. Two weeks after fusion, the hybridomas secreting antibody reactive for pure *Ole e 1* were identified by the ELISA technique using a plate coated with the purified allergen. Positive hybrids were cloned by limiting dilution, and then they were selectively expanded. Ascitic fluid was obtained from pristane-treated mice by injection with hybridoma cells. The resulting ascites were precipitated with 50% saturated ammonium sulfate, dialyzed and resuspended in PBS and stored at -20°C. Isotyping of mAbs was determined using a commercial solid-phase enzyme immunoassay (Sigma, St. Louis, Mo., USA) as described by the manufacturer.

Purification and Labelling of mAbs

Purification of mAbs from ascitic fluid was achieved by gel filtration on Sephadex G-200 (IgM), and by affinity chromatography on a Protein-A Sepharose CL-4B column (Pharmacia LKB, Uppsala, Sweden; IgG) as described by Ey et al. [25]. The purity of mAbs was assessed by SDS-PAGE in the presence or absence of β -mercaptoethanol in 15% homogeneous acrylamide gels, according to the method of Laemmli [23]. The protein concentrations of the purified mAbs were determined from the OD at 280 nm (using $\epsilon_{280}^{1\%1\text{cm}} = 13.8$ for mouse IgG and $\epsilon_{280}^{1\%1\text{cm}} = 11.85$ for mouse IgM).

The mAbs were labelled with horseradish peroxidase (HRPO) according to the procedure of Farr and Nakane [26]. Briefly 1 μg of purified antibody was labelled with 2 μg of HRPO (Sigma, St. Louis, Mo., USA) with a specific activity of 288 units/mg solid. The labelling efficiency was about 55% for all mAbs.

Epitope Mapping

The procedure used was competitive ELISA inhibition. Plates were coated overnight with pure *Ole e 1* (1 $\mu\text{g}/\text{well}$) at 4°C. Peroxidase-labelled purified mAbs (at a dilution equivalent to 70% of maximal binding) were incubated alone or mixed with different concentrations of purified mAbs. The inhibition binding percentage of the peroxidase-labelled antibody to *Ole e 1* was calculated according to the following formula: $[1 - (\text{OD in presence of mAb competitor}/\text{OD in absence of competitor})] \times 100$.

A nonrelated mAb (α -DNP mAb) was used as the negative control.

Identification of Allergenic Determinants

Plates were coated with pure *Ole e 1* as described above. A pool of sera (1/10 diluted on PBS) alone or mixed with different concentrations of purified mAbs (0.1, 1, 10, 50, 100, 200 and 400 $\mu\text{g}/\text{ml}$) was incubated for 3 h at room temperature. The plate was incubated with β -galactosidase-labelled human anti-IgE (Pharmacia, Uppsala, Sweden). The assay was developed using a commercial kit (Phadebas RAST, Pharmacia, Sweden) following the instructions of the manufacturer. As the negative control, we used a nonrelated mAb (anti-DNP ascites).

Reactivity of mAbs with Oleaceae Pollens

This was performed according to the method of Chapman et al. [27]. The antigenic extracts were bound to microtiter plates at 4 $\mu\text{g}/\text{well}$. After incubation with purified mAbs against *Ole e 1* (5 $\mu\text{g}/\text{well}$), HRPO-labelled rabbit anti-mouse immunoglobulins (The Binding Site, Birmingham, UK) were added. To develop the reaction, o-phenylenediamine $\cdot 2\text{HCl}$ (OPD, Sigma) substrate solution was used. We used a nonrelated mAb (α -DNP) and protein (casein) to determine nonspecific binding.

Immunoblot

Electrophoresis was made according to the procedure of Laemmli [23] using 12 and 15% polyacrylamide homogenous gels with 5% stacking gels. The mobility of the bands was measured as described by Weber and Osborn [28].

Electrophoretic blotting of the crude extract from the SDS-PAGE was carried out according to the method of Towbin et al. [29] and modified by Sutton et al. [30]. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories).

In the case of mAb binding assay, the nitrocellulose membrane was incubated with 10 ml of mAbs from ascitic fluid (60 $\mu\text{g}/\text{ml}$) and finally

incubated in a 1:1,000 dilution of HRPO-labelled goat anti-mouse IgG, A. M (H+L; The Binding Site, Birmingham, UK). The blot was developed by using 4-chloro-1-naphthol (Sigma) as substrate.

When the human IgE-binding assay was performed, blots were incubated with a pool of sera diluted 1:10 in TBS-BSA 0.1% and anti-human IgE conjugated with β -galactosidase (Pharmacia, Uppsala, Sweden) diluted 1:10 in TBS-BSA 0.1%. Bound antibodies were detected using β -naphthyl- β -D-galactopyranoside/dianisidine tetrazolium substrate (Sigma).

Ultrastructural Immunolocalization of Ole e 1

Mature anthers of *O. europaea* were dissected from floral buds and fixed in 3% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in a 0.1 M-phosphate buffer (pH 7.2) for 2 h at room temperature. The anthers were dehydrated in a series of ethanol, gradually transferred to propylene oxide and embedded in Epon.

Ultrathin sections (80 nm) were obtained with a Reichert-Jung ultramicrotome (Ultracut E, Reichert-Jung, FRG) and transferred to 300-mesh copper grids.

Copper grids with ultrathin sections were washed with 5% BSA in PBS buffer for 10 min. This was followed by incubation with the mAb diluted to 1:10 in PBS at 37°C for 1 h. After washing in PBS the grids were treated with goat anti-mouse IgG:10 nm gold (Biocell Laboratories) diluted 1:50 in PBS for 45 min at 37°C. Then they were washed in PBS, followed by rinsing in double-distilled water, and stained for 10 min in uranyl acetate. The observation was done with a Zeiss 10C transmission electron microscope. Control was carried out by omitting the mAb step.

Results

Epitope Specificity of mAbs

The mAbs directed against the *Ole e 1* major allergen were produced, isotyped and purified as described in Materials and Methods: 1D7 (IgG₁), 1D8 (IgG₁), 10H1 (IgG_{1a}), 16G2 (IgM), 19G6 (IgG₁) and 21F5 (IgG_{2a}).

Figure 1 shows the competitive inhibition of each mAb by itself and with other mAbs. These studies enabled the identification of four groups of mAbs reacting with different epitopes in *Ole e 1*. Each mAb is inhibited by itself. The 1D8 antibody is completely inhibited by 1D7 and the inhibition is over 20% by 10H1 and by 19G6; 10H1 is inhibited by 19G6; 16G2 is partially inhibited (up to 30%) by 21F5; 21F5 is partially inhibited by 10H1. The 1D7 and 19G6 mAbs are completely inhibited by 1D8 and 10H1, respectively (data not shown). Thus, we classified the mAbs into four different groups: G1 (1D7, 1D8); G2 (10H1, 19G6); G3 (16G2), and G4 (21F5). Inhibition values below 20% have been considered to be nonrelevants. No inhibition was seen with mAbs of different specificity (anti-DNP) which ensures the specificity of the assay.

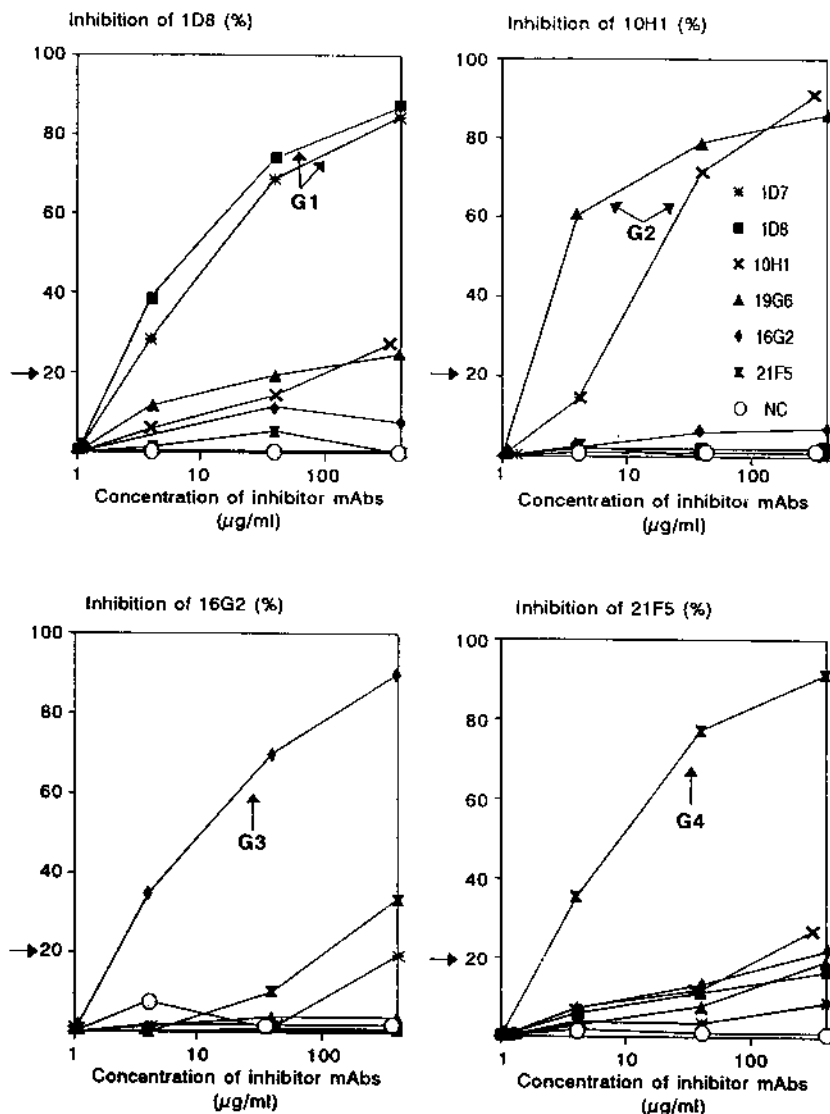


Fig. 1. Determination of epitope specificities of anti-*Ole e 1* mAbs by competitive inhibition assay. The plates were coated with 100 μ l of 10 μ g/ml of pure *Ole e 1*. Each HRPO-labelled mAb (diluted as in Material and Methods) was incubated alone or mixed with other purified mAbs (4, 40 and 200 μ g/ml). Values below 20% (arrows) were considered as not relevant. G1, G2, G3 and G4 correspond to different monoclonal reactivity groups. The results are an average of three different experiments. The standard deviation values were below 6% in all cases. We used a nonrelated mAb (α -DNP) as negative control.

IgE-Binding Inhibition by mAbs

mAbs were used to inhibit the IgE binding to pure *Ole e 1* using a pool of sera from 10 allergic patients sensitized to *O. europaea* pollen ($OD_{492\text{nm}} = 1.5$). As can be seen in figure 2, the maximal inhibition obtained with each mAb was: 0.5% with 21F5 (G4); 7% with 1D8 (G1); 10% with 16G2 (G3), and approximately 18–25% with 10H1 and 19G6 (G2). No inhibition was produced by the mAb α -DNP (negative control).

Reactivity of mAbs with Oleaceae Pollens

ELISA reactivity of these purified mAbs against the pollen of five species of the Oleaceae family is shown in figure 3. We obtained three reactivity patterns: G1 group only recognizes *O. europaea* and *F. excelsior* pollens; G2 reacts with *O. europaea*, *F. excelsior*, *L. vulgare* and *S. vulgaris* pollens, and finally, G3 and G4 recognize all five pollen extracts. The reactivity found with some mAbs is higher with other Oleaceae pollen than with olive pollen. The results indicate that *O. europaea* and *F. excelsior* share at least four epitope determinants (they are reactive with G1, G2, G3 and

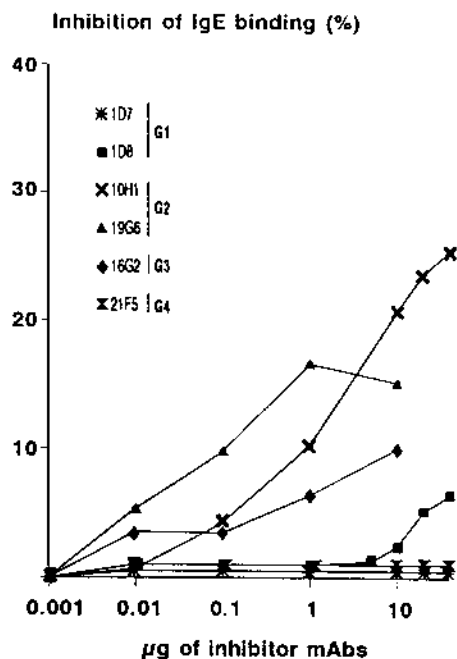


Fig. 2. ELISA inhibition assay of the binding of IgE from allergic sera to *Ole e I* produced by mAbs. The plates were coated with pure *Ole e I* (10 µg/ml). A pool of sera (1/10 diluted) from patients sensitized to *Ole e I* was incubated with different amounts of purified mAbs (0.1, 1, 10, 50, 100, 200 and 400 µg/ml) and thereafter the mixture was tested for IgE anti-*Ole e I* binding. As negative control a nonrelated mAb was used. G1-4 represent different reactivity groups. The results are an average of three different assays. The intra- and interassay variability was below 5%.

G4); *O. europaea*, *L. vulgare* and *S. vulgaris* share three different epitope determinants (G2, G3 and G4), whereas *O. europaea* and *F. suspensa* are recognized by G3 and G4. Because these groups of mAbs recognize different determinants on *Ole e I*, it is interesting to know how these determinants are distributed on the protein pattern of the different Oleaceae family pollens. Protein staining of electrophoretically separated olive, ash, privet, lilac and forsythia pollens revealed a complex band pattern, from 10 to 200 kD MW with intense staining in the 17-, 20- and 40-kD regions (fig. 4A). Blotting of the protein pollen was probed with two mAbs (10H1 and 16G2). The 10H1 antibody reacts with 18-, 20- and 40-kD proteins of olive, ash, privet and lilac (fig. 4B). The 16G2 antibody reacts with 20- and 18-kD proteins of olive, ash, privet and lilac, and with a 50- to 55-kD protein of forsythia (fig. 4C). Other mAbs (G1, and G4) only react with *Ole e I* by ELISA but not by immunoblotting in denatured conditions (data not shown).

Figure 4D shows reactive proteins from the five pollens tested with a pool of sera from olive-allergic patients. A very similar reactivity profile was found for all the pollens, since the same two bands of 18 and 20 kD were presented on the different pollens, except in the case of forsythia which has a 50- to 55-kD reactive band.

Ultrastructural Immunolocalization of Ole e I Molecule in the Pollen Grain

After incubation with mAb 10H1, gold particles were only observed in the cytoplasm, whereas the vegetative nucleus, generative cell, pollen grain wall and apertural regions were not labelled (fig. 5A). Gold particles in the cytoplasm were associated with the dilated endoplasmic reticulum cisterns while organelles such as mitochondria, plastids and cytoplasmic vesicles were devoid of gold particles (fig. 5B). No significant labelling was present in the control (fig. 5C).

Discussion

Ole e I has at least four epitope determinants in its molecule identified by competition assays with six mAbs (fig. 1). It is known that the entire surface of a protein molecule is potentially antigenic and frequently antibodies partially overlap their recognition surfaces. Furthermore, binding of one of the antibodies may cause conformational changes in the molecule, in such a way that the second antibody is unable to recognize it [31]. In our case, mAbs which belong to G2 (10H1 and 19G6) partially inhibit (up to 20%) the binding of mAbs from G1 and G4, and G4 (21F5) inhibits the binding of G3 to the molecule by over 30%. However, the binding of G2 to *Ole e I* is not inhibited by the other mAbs. This suggests that G2 recognizes neighboring areas with respect to those recognized by G1 and G4 on the *Ole e I* molecule, and G4 acts in the same way with respect to G3 (16G2). Only inhibition values above 20% were considered because, for epitope definition, high binding inhibition values of mAbs should be required.

It is known that the detection of different epitopes have to be defined based on the technique employed [32]. Different techniques could give rather different results, for this reason we used catching ELISA to validate our technique. The results of epitope mapping by catching ELISA with G2 group mAbs (10H1 and 19G6) were coincidental with those described above (fig. 1), that is, only 10H1 completely inhibited the binding of 19G6 to *Ole e I* and vice versa (data not shown).

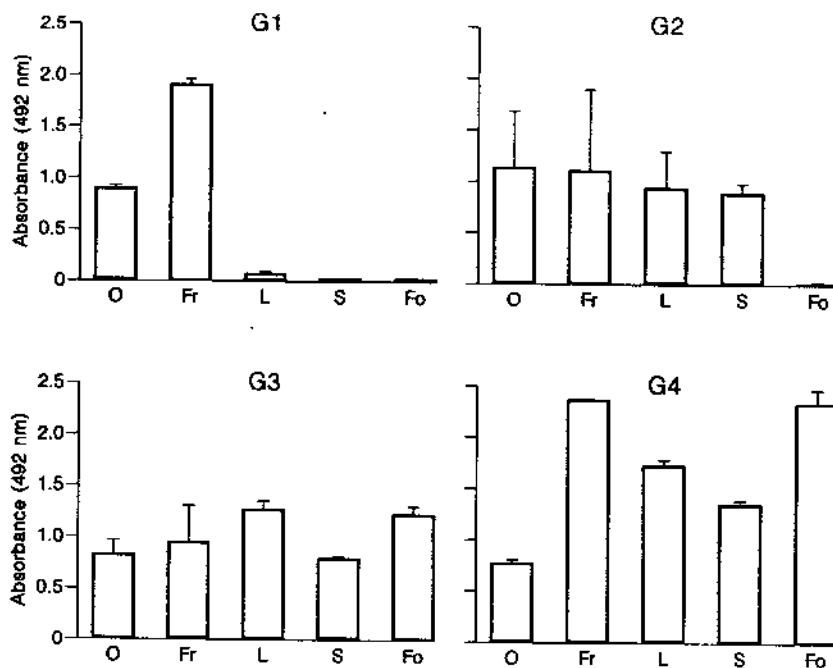


Fig. 3. Reactivity by ELISA of the purified mAbs anti *Ole e I* (5 μ g/well) with the five Oleaceae pollen extracts (4 μ g/well). The extracts used were: olive pollen extract (O); ash pollen extract (Fr); privet pollen extract (L); lilac pollen extract (S) and forsythia pollen extract (Fo). The different mAb reactivity groups are represented by G1-4. Four different experiments were made. The specificity of the reaction was assessed with a nonrelated mAb anti-DNP and by a nonrelated antigen (casein); the absorbance values being below 0.05 in each case.

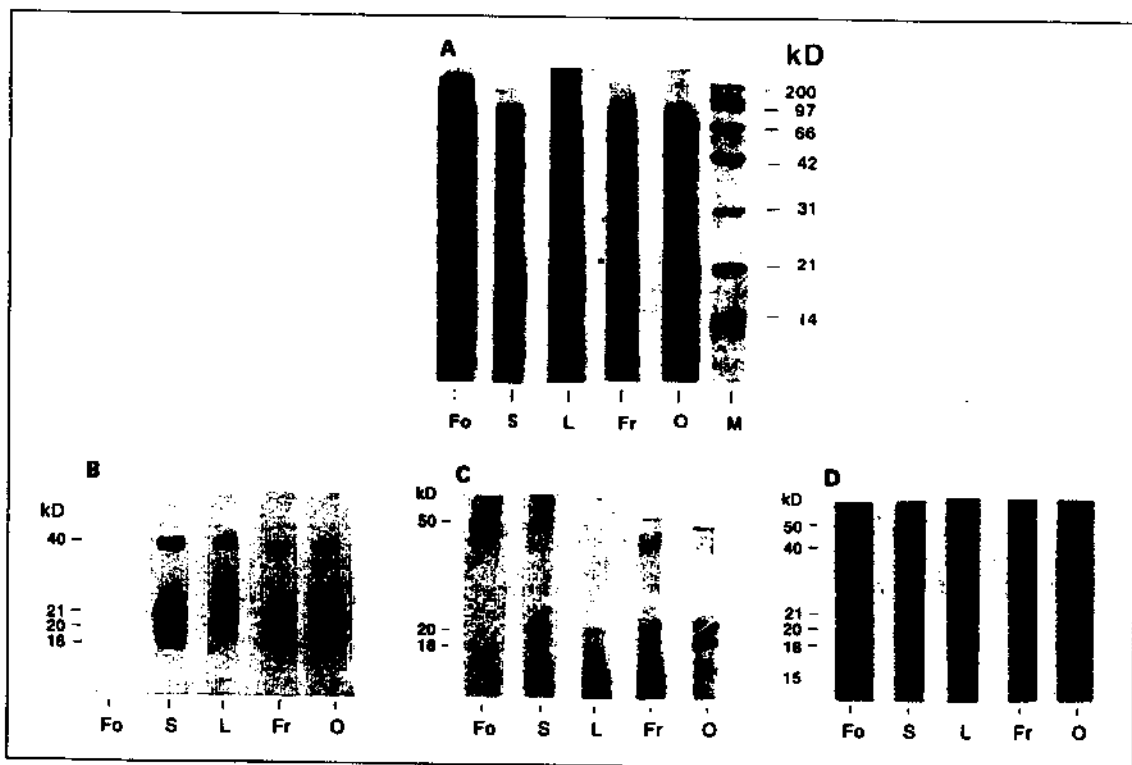
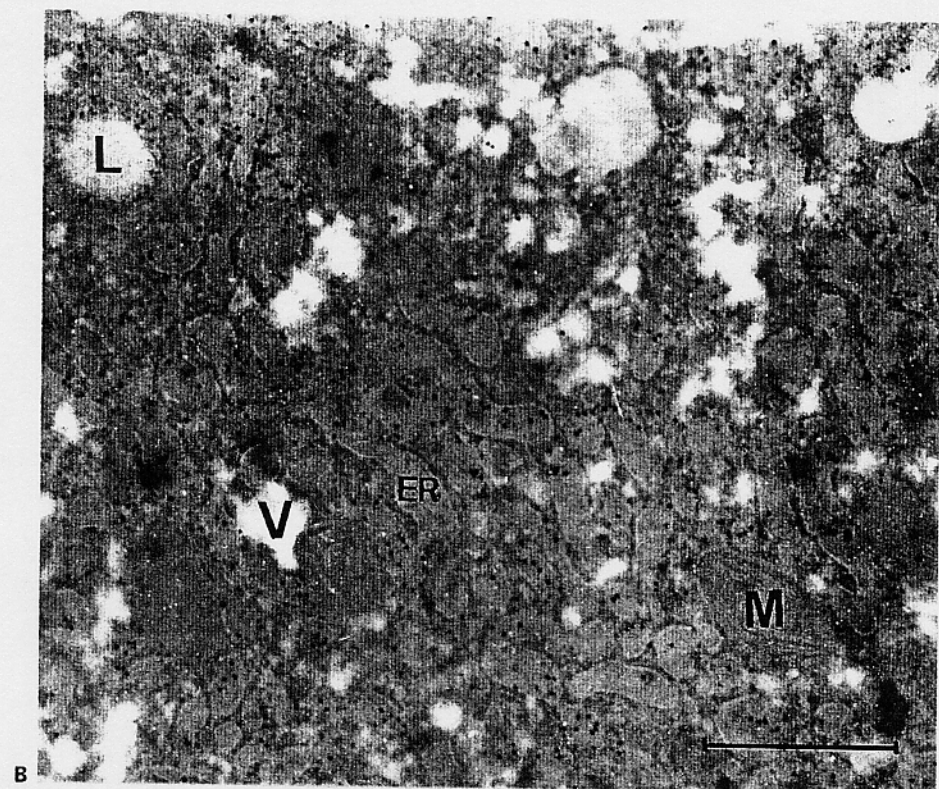


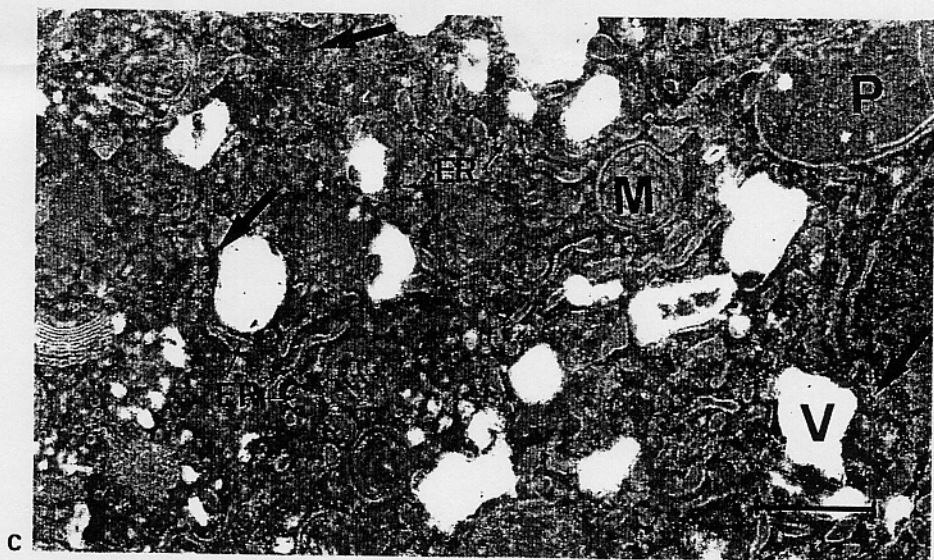
Fig. 4. A SDS-PAGE of the five Oleaceae pollens. B-D Immunoblot profiles of the five Oleaceae pollen extracts separated by SDS-PAGE and incubated with: 10H1 (B), 16G2 (C) and a pool of allergic sera (D). B, C developed with peroxidase-labelled anti-mouse immunoglobulins. D Developed with β -galactosidase-labelled anti-human IgE. M = Molecular weight markers; Fo = forsythia pollen extract; S = lilac pollen extract; L = privet pollen extract; Fr = ash pollen extract; O = olive pollen extract.



Fig. 5. A Mature pollen grain of *O. europaea* after incubation with mAb 10H1. Gold particles are only located in the cytoplasm (Cy). The vegetative nucleus (VN), generative cell (GC), pollen wall (PW), plastids (P) and apertural region (A) are devoid of gold particles. Bar represents 1 μ m.



B



C

Fig. 5. B Detail from the cytoplasm of *O. europaea* mature pollen grain after immunocytochemical localization using 10H1 mAb. Gold particles are concentrated in dilated endoplasmic reticulum cisternae (ER). The absence of particles in mitochondria (M), vesicles (V) and lipid bodies (L) should be noted. Bar represents 0.5 μ m. **C** Mature pollen grain of *O. europaea* showing part of the cytoplasm. Incubation with PBS buffer instead of mAb 10H1 (control). Only a few nonspecifically bound gold particles can be observed (large arrows). Bar represents 0.5 μ m.

The capacity of these epitopes to be recognized also by human allergen-specific IgE antibodies was assessed by binding inhibition assay (fig. 2). It was clearly shown that the human IgE-binding inhibition to pure *Ole e 1* by mAbs, ranged from 0.5 to 25%. This small effect on IgE inhibition by mAbs may be because human IgE antibodies are directed against multiple epitope determinants and in the case of mAbs only one epitope is blocked. This explanation agrees with the data of Chapman et al. [27], who found that individual mAbs against two distinct epitopes on *Der p 1* allergen from *Dermatophagoides pteronyssinus* had very little effect on human specific IgE binding, while specific IgE from human sera totally inhibited the binding of the mAbs. In our case, the specific IgE-binding inhibition with G1, G2 and G3 suggests that these mAbs recognize allergenic determinants, while 21F5 (G4), which failed on IgE inhibition, could react with an antigenic determinant or with an allergenic determinant poorly represented on the *Ole e 1* molecule.

We have found that the epitopes of *Ole e 1* are present not only on *Olea*, but also on proteins of related species from the Oleaceae family such as ash (*F. excelsior*), privet (*L. vulgare*), lilac (*S. vulgaris*) and forsythia (*F. suspensa*; fig. 3, 4). Although the sequences from the N-terminal end of *Ole e 1* and the '*Ole e 1*-like' proteins obtained from ash, privet and lilac have been reported to be identical [9], our results indicate the existence of some differences in immunological behavior. We found a heterogeneous recognition pattern and reactivity of the anti-*Ole e 1* mAbs with respect to the Oleaceae pollens. Two points should be considered: first, the identity found in the N-terminal end sequence among the different members of the family is surprising [9] since the data from Villalba et al. [7] point to a certain degree of heterogeneity in the *Ole e 1* amino acid sequence. Ibrahim et al. [33] studied four closely related lysozymes using anti-lysozyme and anti-loop antibodies, and reported that a single amino acid substitution of the loop region of lysozyme can affect its antigenic specificity. Second, if the similarity among the allergens in the Oleaceae family was as close as suggested by Obispo et al. [9], one would expect a major incidence of allergies produced by lilac, privet, etc., which is not the case. Although this second point could be explained by the greater pollen size of these species in respect to olive and ash and by the differences on pollination methods: by insects (entomophilous) or by wind (anemophilous) [34].

The reactivity by ELISA of some mAbs is higher with the other pollens than with olive (fig. 3). Harper et al. [31] reported that 19 of 27 anti-hen eggwhite lysozyme mAbs bind heterologous egg lysozymes from turkey, pheasant and Guinea hen with somewhat higher association constants

than those found for the homologous antigen (hen eggwhite lysozyme). This increase in affinity may be explained by a small increase in the number of Van der Waals or hydrogen-bonded interactions, resulting from one or a small number of amino acid replacements in the antigen. Somatic mutations in the antibody genes could also introduce new interactions giving rise to a similar increase in affinity [31].

The immunoblotting of the pollens was developed with a pool of sera and with different mAbs. G2 and G3 (10H1 and 16G2) recognized the pollens by ELISA (fig. 3) and immunoblotting after SDS-PAGE (fig. 4B, C), whereas G1 and G4 (1D7, 1D8 and 21F5) recognized the pollens by ELISA but not on immunoblot. This could be explained by the existence of conformational epitopes that are recognized by these mAbs only under nondenatured conditions. Thus, it has been known that more than a third, and perhaps a majority of the antibodies obtained by immunizing with native protein, react against assembled topographic sites [35].

The results of pollen immunoblotting (fig. 4B-D) show two proteins with MWs of 18 and 20 kD (which correspond with the MW of the two polypeptides of *Ole e 1*) as one of the main causes of cross-reactivity among olive, ash, privet and lilac. In contrast with the results of Obispo et al. [9], other bands of 22 and 40 kD are also implicated. Even in the case of forsythia (fig. 4C, D) the only reactive band was in the MW range of 50-55 kD. This result could be explained because 18- and 20-kD polypeptides could be present in forsythia as aggregates of 50-55 kD. These data, in agreement with the work of Vela et al. [2] and Wheeler et al. [6], indicate that native *Ole e 1* could be a multimeric structure or aggregation product in which the 18- and 20-kD polypeptides are associated in some way. We have found that the epitope determinants on the Oleaceae family have been conserved. So, the reactivity studies with different groups of mAbs appear to indicate a high degree of similarity among members of the Oleaceae family.

The analysis of the subcellular distribution of the *Ole e 1* antigen, using G2 (10H1 mAb) in mature olive pollen grain, allows the following conclusion to be drawn. *Ole e 1* is located in association with the endoplasmic reticulum cisternae in the cytoplasm. In a previously study [36], the outstanding proliferation that undergoes the endoplasmic reticulum cisternae during the maturation of the olive pollen grain, was shown. However, in the case of ryegrass mature pollen grain [20], the allergen was located in the electron opaque areas of the cytoplasm, especially mitochondria, and in birch pollen grain [21] in the cytoplasmic matrix. As far as we know, this is the first time that the location of an allergen in cell organelles such the endoplasmic reticulum or the Golgi apparatus has been reported.

In summary, this study makes the following points. (1) *Ole e I* presents at least four epitope determinants in its molecule. (2) Three of these epitopes are recognized by a pool of allergic sera (allergenic epitopes). (3) Several antigenic determinants from *Ole e I* are present to different degrees on other proteins from the Oleaceae family. It seems that the Oleaceae pollens present homologous proteins with MWs of 18 and 20 kD, which may differ in a small number of substitutions in their sequence which could modify their allergenic structure. (4) *Ole e I* is located in the endoplasmic reticulum cisternae in the pollen grain cytoplasm.

Acknowledgments

We are grateful to Dr. Ortiz Masllorens for continuous support. We also thank Ms. Paloma Tramón for skilful technical assistance.

This work was supported by grants from: Comunidad Autónoma de Madrid C 103/90, Fondo de Investigaciones Sanitarias de la Seguridad Social: 90/238 and Dirección General de Investigación Científica y Técnica-DGICYT (project N°. PB92-0079-C03-03).

References

- Bousquet J, Guerin B, Hewitt B, Lim S, Michel FB: Allergy in the Mediterranean area III: Cross-reactivity among Oleaceae pollens. *Clin Allergy* 1985;15:439-448.
- Vela C, Platas C, Gurbindo C, Tricas L, Subiza E, García R, Lahoz C: Fractionation and biological characterization of *Olea europaea* pollen extract. *Int Arch Allergy Appl Immunol* 1982; 68:289-294.
- Bianca M, Bulton P, Brostoff J, Gonzalez-Reguera I: Studies of the allergens of *Olea europaea* pollen. *Clin Allergy* 1983;13:473-478.
- Lauzurica P, Gurbindo C, Maruri N, Galocha B, Díaz R, González J, García R, Lahoz C: Olive (*Olea europaea*) pollen allergens. I. Immunochemical characterization by immunoblotting, CRIE and immunodetection by a monoclonal antibody. *Mol Immunol* 1988;25:329-335.
- Lauzurica P, Maruri N, Galocha B, González J, Díaz R, Palomino P, Hernández D, García R, Lahoz C: Olive (*Olea europaea*) pollen allergens. II. Isolation and characterization of two major antigens. *Mol Immunol* 1988;25:337-344.
- Wheeler AW, Hickman BE, Fox B: Heterogeneity of a major allergen from olive (*Olea europaea*) pollen. *Mol Immunol* 1990;27:631-636.
- Villalba M, López-Otin C, Martín-Orozco E, Monsalve RI, Palomino P, Lahoz C, Rodríguez R: Isolation of three allergenic fractions of the major allergen from *Olea europaea* pollen and N-terminal amino acid sequence. *Biochem Biophys Res Commun* 1990;172:523-528.
- Lombardero M, Quirce S, Duffort O, Barber D, Carpizo J, Chamorro MJ, Lezaun A, Carreira J: Monoclonal antibodies against *Olea europaea* major allergen: Allergenic activity of affinity-purified allergen and depleted extract and development of a radioimmunoassay for the quantitation of the allergen. *J Allergy Clin Immunol* 1992;89:884-894.
- Obispo TM, Melero JA, Carpizo JA, Carreiro J, Lombardero M: The main allergen of *Olea europaea* (*Ole e I*) is also present in other species of the Oleaceae family. *Clin Exp Allergy* 1993; 23:311-316.
- Villalba M, Batanero E, López-Otin C, Sánchez LM, Monsalve RI, González de la Peña MA, Lahoz C, Rodríguez R: The amino acid sequence of *Ole e I*, the major allergen from olive tree (*Olea europaea*) pollen. *Eur J Biochem* 1993;216:863-869.
- King TP: Chemical and biological property of some atopic allergens. *Adv Immunol* 1976;23: 77-105.
- Ipsen H, Bowadi H, Janniche H, Petersen BN, Munch EP, Wihl JA, Löwenstein H: Immunochemical characterization of reference alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen extracts and the partial immunochemical identity between the major allergens of alder, birch and hazel pollens. *Allergy* 1985;40:510-518.
- Weber RW, Nelson HS: Pollen allergens and their interrelationship. *Clin Rev Allergy* 1985; 3:291-318.
- Kenerman SM, McCullough J, Green J, Ownby DR: Evidence of cross-reactivity between olive, ash, privet and Russian olive tree pollen allergens. *Ann Allergy* 1992;69:493-496.
- Baldo BA, Panzani RC, Bass D, Zerboni R: Olive (*Olea europaea*) and privet (*Ligustrum vulgare*) pollen allergens. Identification and cross-reactivity with grass pollen proteins. *Mol Immunol* 1992;29:1209-1218.
- Shen H-D, Chan LY, Su SN, Han SH: Characteristics of five monoclonal antibodies to major allergens of the short ragweed pollen. *Int Arch Allergy Appl Immunol* 1988;85:167-173.
- Knox RB, Vithanage HIMV, Howlet BJ: Botanical immunocytochemistry: A review with special reference to pollen antigens and allergens. *Histochem J* 1980;12:247-272.
- Vithanage HIMV, Howlet BJ, Jobson S, Knox RB: Immunocytochemical localization of water-soluble glycoproteins, including group allergen in pollen of ryegrass, *Lolium perenne*, using ferritin-labelled antibody. *Histochem J* 1982;14:949-966.
- Grote M, Fromme HG: Cross-reactivity of birch anther and leaves with birch pollen antigens and allergens. A fine-structural immunocytochemical study using the post-embedding protein-A-gold technique. *J Histochem Cytochem* 1986; 34:1459-1464.
- Staff JA, Taylor PE, Smith P, Sing MB, Knox RB: Cellular localization of water soluble allergenic proteins in rye-grass (*Lolium perenne*) pollen using monoclonal and specific IgE antibodies with immunogold probes. *Histochem J* 1990;22:276-290.
- Grote M: Immunogold electron microscopy of soluble proteins: Localization of *Bet v I* major allergen in ultra-thin sections of birch pollen after anhydrous fixation techniques. *J Histochem Cytochem* 1991;39:1395-1401.
- Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein. *Anal Biochem* 1976;72:248-254.
- Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond)* 1970;227:680-685.
- Köhler G, Milstein C: Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* 1976;6:511-519.
- Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG1, IgG2a, IgG2b immunoglobulins from mouse serum using protein-A Sepharose. *Immunochimistry* 1978;15:429-436.
- Farr AG, Nakane PK: Immunochimistry with enzyme antibodies: A brief review. *J Immunol Methods* 1981;47:129-144.
- Chapman M, Sutherland W, Platts Mills T: Recognition of two *Dermatophagoides pteronyssinus* specific epitopes on antigen PI by using monoclonal antibodies. Binding to each epitope can be inhibited by serum from dust mite-allergic patients. *J Immunol* 1984;133:2488-2495.

- 28 Weber K, Osborn M: The reliability of molecular weight determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J Biol Chem* 1969;244:4406-4412.
- 29 Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350-4354.
- 30 Sutton R, Wrigley CW, Baldo BA: Detection of IgE and IgG binding proteins after electrophoretic transfer from polyacrylamide gels. *J Immunol Methods* 1982;52:183-194.
- 31 Harper M, Lema F, Boulot G, Poljak J: Antigen specificity and cross-reactivity of monoclonal anti-lysozyme antibodies. *Mol Immunol* 1987; 24:97-108.
- 32 Almondalal ZA, Briand JP, Van Regenmortel MHV: A major part of the polypeptide chain of tobacco mosaic virus protein is antigenic. *EMBO J* 1985;4:1231-1235.
- 33 Ibrahim IM, Eder J, Prager EM, Wilson AC, Arnon R: The effect of a single amino acid substitution on the antigenic specificity of the loop region of lysozyme. *Mol Immunol* 1980;17: 37-46.
- 34 Platts-Mills TAE, Solomon WR: Acrobiology and inhalant allergens; in Middleton E Jr, Reed CE, Ellis EF, Adkinson NF Jr, Yunginger JW, Busse WW (eds): *Allergy. Principles and Practice*, ed 4. St. Louis, Mosby, 1933, pp 469-489.
- 35 Berzofsky JA: Intrinsic and extrinsic factors in protein antigenic structure. *Science* 1985;229: 932-940.
- 36 Rodríguez-García MI, Fernández MC: Ultrastructural evidence of endoplasmic reticulum changes during the maturation of the olive pollen grain (*Olea europaea* L., *Oleaceae*). *Plant Syst Evol* 1990;171:221-231.